Supporting Information (SI)

JNK regulates the photic response of the mammalian circadian clock

Hikari Yoshitane, Sato Honma, Kiyomichi Imamura, Hiroto Nakajima, Shin-ya Nishide, Daisuke Ono, Hiroshi Kiyota, Naoya Shinozaki, Hirokazu Matsuki, Naoya Wada, Hirofumi Doi, Toshiyuki Hamada, Ken-ichi Honma and Yoshitaka Fukada

SI Materials and Methods

Cell culture and transfection

NIH3T3 cells, Rat1 cells, HEK293T cells and PER2::Luc MEF (1) were maintained at 37°C under 5% CO_2, 95% air in Dulbecco's modified Eagle's medium (Nissui) containing 1.8 mg/ml $NaHCO_3$ and 4.5 mg/ml glucose supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. For detection of endogenous BMAL1 in NIH3T3 cells, the cells in 10-cm dish were washed with PBS, and were solubilized in ice-cold IP buffer (20 mM HEPES-NaOH, 137 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM DTT, 1 mM PMSF, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 50 mM NaF, and 1 mM Na_3VO_4; pH 7.8). The cell extracts were then centrifuged for 10 min, 21,600 ×g, and the supernatant was subjected to immunoprecipitation with CLNT1 anti-CLOCK mAb and Protein G-Sepharose. For transient transfection, the cells were plated in 12-well plates 24 hr before the experiments, and were transiently transfected by using Lipofect AMINE PLUS Reagent (Invitrogen) according to manufacturer's directions. Total amount of transfected DNA was kept constant in an experiment by adding the empty plasmid. For activation of JNKs, the cells were treated for 30 min with sorbitol just before harvesting the cells. Cells were washed with PBS 36 hr after the transfections, and solubilized in RIPA (50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 50 mM NaF, and 1 mM Na_3VO_4; pH 8.0). The cell extracts were then centrifuged for

10 min, 21,600 ×g, and the supernatant was subjected to immunobolotting.

Plasmid

The mammalian expression vectors used were Myc-CLOCK/pSG5 (a kind gift of Dr. Paolo Sassone-Corsi), BMAL1/pcDNA3.1 (a kind gift of Dr. Steve M. Reppert), and Flag-JNK1/pSRα (a kind gift of Dr. Mutsuhiro Takekawa). Ala mutations were introduced into BMAL1 by using a site-directed PCR mutagenesis method. Full-length mouse *Jnk1α1*, *Jnk2α1* and *Jnk3α1* cDNA were cloned from the total cDNA of the mouse SCN (C57BL/6J) by RT-PCR analysis with gene-specific primers. The primers used were: *Jnk1α1* Fw; 5' -GCGGC CGCAT GAGCA GAAGC AAACG TG- 3', *Jnk1α1* Rv; 5' -GCGGC CGCTC ATTGC TGCAC CTGTG C- 3', *Jnk2α1* Fw; 5' -GCGGC CGCAT GAGTG ACAGT AAAAG CGATG- 3', *Jnk2α1* Rv; 5' -GCGGC CGCTT ACTGC TGCAT CTGTG CTG- 3', *Jnk3α1* Fw; 5' -GCGGC CGCAT GAGCC TCCAT TTCTT ATAC- 3', *Jnk3α1* Rv; 5' -GCGGC CGCTC ACTGC TGCAC CTGTG C- 3'. Mammalian expression vectors for expression of Flag epitope-tagged JNK isoforms were generated by inserting the *Jnk* cDNAs into the pSG5 vector, with a slight modification to create NotI sites, a Kozak sequence, and a Flag epitope. Full-length human *Mkk7, Jnk1α1* and *Jnk3α1* cDNA were cloned and inserted into the pCI vector for Flag-MKK7-JNK1/pCI and Flag-MKK7-JNK3/pCI. Lys93 in JNK3 was mutated to Ala as a kinase-dead mutant. For knockdown of JNK1 and JNK2, shRNA were designed using siDirect (http://sidirect2.rnai.jp/), a web-based software, and the following sequence was used: shJNK1; 5' -GAGAA CUAGU UCUUA UGAAG U- 3', shJNK2; 5' -GUAUA UUACU GUUUG GUAUG A- 3', and shJNK1/2; 5' -GGAAU AAAGU UAUUG AACAG C- 3'. The oligonucleotides to express the shRNA were inserted into the pBS-mU6 vector (modified by Dr. Kimiko Shimizu from pBS-hU6 vector)(2).

Antibodies, Immunoprecipitation and immunoblot analysis

Anti-CLOCK monoclonal antibody (mAb), CLNT1, was used for immunoprecipitation

as described in our previous paper (3). In immunoblot analysis, antibodies used were CLSP3 anti-CLOCK mAb (3), B1BH2 anti-BMAL1 mAb (3), anti-JNK1/3 (Santa Cruz Biotechnology, C17), anti-JNK2 (Millipore), anti-JNK3 (Millipore, C05T), anti-phospho-JNKs (Cell Signaling) and anti-β-actin (Sigma Aldrich). For specific detection of JNK1, anti-JNK1 (Santa Cruz Biotechnology, F3) was used in Fig. 5A and Fig. S7. The bound primary antibodies were detected by HRP (horseradish peroxidase)-conjugated anti-rabbit or anti-mouse IgG antibody (Kirkegaard & Perry Laboratories).

Preparation of nuclear proteins

The nuclear proteins and cytoplasmic proteins were prepared as described (3). Briefly, the tissue (1 g wet weight) was washed with ice-cold PBS and homogenized at 4°C with 9 ml of ice-cold buffer A (10 mM HEPES-NaOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 4 μg/ml aprotinin, 4 μg/ml leupeptin, 50 mM NaF, and 1 mM Na_3VO_4). The homogenate was centrifuged twice (5 min each, 700 ×g), and the unsedimented material was collected as a “cytoplasmic fraction”. The precipitate was resuspended in 2 ml of ice-cold buffer C (20 mM HEPES-NaOH (pH 7.8), 400 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 2% (v/v) glycerol, 1 mM DTT, 1 mM PMSF, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 50 mM NaF, and 1 mM Na_3VO_4). After being gently mixed at 4°C for 30 min, the suspension was centrifuged twice (30 min each, 21,600 ×g), and the final supernatant was used as a “nuclear extract”.

Real-time monitoring assay

Real-time monitoring assay was performed as described (4) with minor modifications. NIH3T3 cells plated on 35-mm dishes were transiently transfected by shRNA vectors with *Bmal1*-luc/pGL4*,* a firefly luciferase reporter under regulation of a 0.3-kb *Bmal1* promoter. The cells were treated with 0.1 μM dexamethasone for 2 h, and then the medium was replaced by a recording medium [phenol-red free DMEM (Sigma Aldrich)

supplemented with 10% FBS, 3.5 g/l glucose, 25 U/ml penicillin, 25 μg/ml streptomycin, 0.1 mM luciferin (Promega), and 10 mM HEPES-NaOH; pH7.0]. Bioluminescence was recorded for 1 min at 9-min intervals for 5-10 days at 37ºC in air with Dish Type Luminescencer, Kronos (ATTO, AB-2500) or LumiCycle (Actimetrics). NIH3T3 cells and Rat1 cells that stably expressed a firefly luciferase reporter under regulation of a 0.3-kb *Bmal1* promoter was used in Fig. S3A. PER2::Luc MEF (1) was used in Fig. S3B. Raw data of the rhythms were smoothed by 2-hr moving averages, and the highest or lowest level of the bioluminescence signals in each cycle was defined as the peak or trough, respectively.

Animals

Jnk3-deficient mice (backcrossed for more than 10 generations on the C57BL/6J background, Jackson Laboratory, Mapk10^{tm1Flv})(5) and wild-type C57BL/6J mice were reared in our animal quarters where the environmental conditions were controlled (room temperature 22 ± 2°C, humidity 60 ± 5%, light-on 6:00-18:00). Animals had free access to commercial chow (Oriental Yeast Co.) and tap water. Experimental procedures involving animals were approved by Animal Research Committee Hokkaido University (Apporoval No. 08-277) and animals were handled in accordance with the Guidelines for the Care and Use of Laboratory Animals at Hokkaido University.

Behavioral rhythm measurement

Male homozygote mice of *Jnk3*-deficient (n = 22) and wild-type control mice (n = 16) were individually housed in a polycarbonate cage at the age of 7 to 8 weeks. They were individually kept in a light-tight, sound-proof activity recording box, and the spontaneous locomotor activity was measured every min by an infrared thermal sensor (6), first in LD 12:12 (light intensity in the light phase 300 lux) for about 2 weeks and then in DD for 8 to 12 weeks. Single light pulses (30 min, 300 lux) were given every 2 to 3 weeks at 6 different circadian times in subjective night phase (CT12,

14, 16, 20, 22 and 24) where the activity onset was designated as CT12. Single mice were exposed to 2-3 pulses of different CTs at least one at early subjective night and the other at late subjective night, except five *Jnk3*-deficient mice which received only one pulse at CT24. In total 42 light pulses were given in each genotype. Behavioral activity rhythms were analyzed by Clock Lab (Actimetrics, IL) as described elsewhere (6). Circadian periods were analyzed by a chi-square periodogram using data of 10 days before every light pulse. Change in the circadian period in DD was examined excluding mice which received only one light pulse. Phase-shifts were calculated on the next day of the light pulse, by calculating the phase-difference between the two regression lines, one fitted to 10 consecutive activity onsets immediately before the light pulse and the other, to those after the light pulse excluding transient periods of 5 days.

Responses of the circadian clock to different light intensities in LL condition were examined in adult male *Jnk3* deficient (n = 5) and wild-type mice (n = 5). After monitoring spontaneous locomotor activity in LD 12:12 for 3 weeks, they were exposed to constant conditions of 4 different light intensities, 0 (DD), 10, 100 and 300 lux for 7, 5, 4 and 4 weeks, respectively. Light intensity was examined inside the cage at 12 cm from the floor. Circadian period was calculated by a chi-square periodogram using the last 10-day records of each lighting condition except for LL at 300 lux. Circadian period in LL at 300 lux was calculated using the data of day 2-11, because three *Jnk3*-deficient mice and one wild-type mouse became arrhythmic in the last 10 days in this condition. In wild-type mice, circadian periods in each light condition were significantly different except those between 10 and 100 lux ($p < 0.01$, One way ANOVA, post hoc Tukey Kramer test), whereas, in *Jnk3*-deficient mice, significant difference was detected only between DD and 300 lux ($p < 0.01$, post hoc test). Significant genotype differences were detected in the light intensity-dependent period increase ($p < 0.01$, Two way ANOVA, light intensities, light x genotype, with pot hoc Tukey Kramer test ** : $p < 0.01$ vs. wild-type mice).

SI References

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Legends to Supplemental Figures

Figure S1: BMAL1 phosphorylation by JNK1 in cultured fibroblasts.

HEK293T cells were cultured in wells of 12-well plates, and transiently transfected with of Myc-CLOCK/pSG5, BMAL1/pcDNA3.1 (WT, mut3A or mut8A) and of JNK1/pSRα. (A) The transfected cells were collected 0, 15, 30 and 60 min after the 600 mM sorbitol stimulation, and the cell lysates were subjected to immunoblot analysis with anti-JNK1 antibody (top panel) or anti-phospho-JNK antibody (bottom panel). Data are means with SEM (n = 3). (B-D) The transfected cells were incubated with 20 μM of SP600125 (SP) or its negative inhibitor (NI) for 2 hr, and then stimulated by sorbitol (600 mM; B, D or 800 mM; C) for 30 min. The collected cell lysates were subjected to immunoblot analysis. Data are means with SEM (n = 3). Double asterisks indicate $p < 0.01$ (Student's *t*-test, vs. WT, 0 mM). Ser17, Thr233, Ser513, Ser520, Thr527, Ser533, Ser545 and Ser592 in mouse BMAL1 were mutated to Ala (mut8A).

Figure S2: *In vitro* phosphorylation of BMAL1 and CLOCK by JNKs.

In vitro phosphorylation of BMAL1 and CLOCK by various isoforms of MAPKs. Activated human JNK1, JNK2, JNK3, ERK1, ERK2 and p38α, all of which are in GST-fusion, were purchased from Carna Bioscience, Inc. (Kobe, Japan). They were incubated with GST-BMAL1 or each of the four regions of GST-CLOCK in 20 μl of the kinase buffer (50 mM HEPES-NaOH, 2 mM DTT and 20 mM $MgCl_2$; pH 7.5) at 30°C for 1 hr in the presence of 10 μM [γ-^{32}P]ATP (111 kBq/pmol). The reaction mixture was then subjected to SDS-PAGE, and the phosphorylation level of the substrate was estimated by autoradiography by using an image analyzer (FLA3000, Fujifilm, Tokyo, Japan). GST and GST-c-Jun(1-79) (Cell Signaling Technology, Inc.) were used as control substrates. (A) GST (1 μg), GST-BMAL1 (0.5 μg), GST-CLOCK(364-537) (0.1 μg), or GST-c-Jun(1-79) (0.15 μg) was incubated with any one of the indicated MAPK: JNK1 (0.15 μg), JNK2 (0.1 μg), JNK3 (0.1 μg), ERK1 (0.07 μg), ERK2 (0.05 μg), or p38α (0.08 μg), in the presence of [γ-^{32}P]ATP. The reaction mixture was then subjected to autoradiography. (B) The indicated GST-fused proteins (0.15 μg each)

were subjected to SDS-PAGE and CBB staining. (C) The indicated GST-fused proteins (0.15 μg each) were incubated with 0.1 μg of JNK3 in the presence of [γ-^{32}P]ATP. The reaction mixture was then subjected to autoradiography.

Figure S3: Effects of JNK inhibitor SP600125 on BMAL1 phosphorylation and cellular rhythms.

(A) Effects of SP600125 on the circadian *Bmal1-luc* rhythms were examined in NIH3T3 cells (top), in Rat-1 fibroblasts (bottom). The NIH3T3 and the Rat-1 cells stably expressing *Bmal1-luc* (4) were synchronized by 2-hr dexamethasone (Dex) treatment, and luciferase activity was monitored in the presence of indicated concentration of SP600125. A representative set of bioluminescence rhythms out of three independent experiments is shown. The periods of the rhythms in NIH3T3 cells are 21.0, 23.2, 24.5 and 26.2 hr with 0, 2.5, 5 and 10 μM SP600125, respectively. (B) PER2::Luc MEFs cultured in 35-mm dishes were synchronized with dexamethasone (Dex). After 2-hr incubation with Dex, the medium was changed to the recording medium (at time 0) that contained the indicated inhibitor. The periods of the rhythms are 22.7, 26.2 and 31.3 hr with 0, 5 and 20 μM SP600125, respectively. The periods of the rhythms are 22.7, 23.8 and 25.0 hr with 0, 5 and 20 μM IC261, respectively. (C) The SCN slices were prepared from adult *Bmal1-luc* mice kept in LD cycles. Bioluminescence rhythms from the SCN slice were recorded as previously described (6). On the 5th day of culture, SP600125 was added at indicated concentrations to the culture media. (D) Circadian periods before and during SP600125 treatment were analyzed using bioluminescence data of 5 consecutive cycles, and expressed in means with SD (n = 5). The periods of the rhythms during the treatment are 23.1, 28.5 and 31.6 hr with 0, 10 and 50 μM SP600125, respectively. (E) After 24-hr incubation of NIH3T3 cells with the indicated inhibitor (0, 5, 20 μM) in 10-cm dish, the cells were collected and total protein extracts were immunoprecipitated with CLNT1 anti-CLOCK mAb, followed by immunoblotting with B1BH2 anti-BMAL1 mAb.

Figure S4: JNK isoforms expressed in NIH3T3 cells and their knockdown constructs.

(A-F) Expression levels of *Jnk* genes were estimated by real-time RCR analysis. Quantification of target gene expression in all samples was normalized to *Tbp* expression by the equation, Ct(target) – Ct(*Tbp*) = ΔCt, where Ct is the threshold cycle number. Changes in target gene expression in each sample were calculated by 2-(ΔCt). (A) Evaluation of PCR efficiency by primers used in real-time RCR. The primers used were, *Jnk1*-Fw TCCCC GATGT GCTTT TCCCA GC, *Jnk1*-Rv TGGGT GCTGG AGAGC TTCAT CTACG, *Jnk2*-Fw GACGA AGCCT TGCGC CACC, *Jnk2*-Rv ATGCT CTCTT TCTTC CAACT GGGC, *Jnk3*-Fw GACGA CGCAC TGCAG CATCC, and *Jnk3*-Rv GGTGT GCTCC CTTTC ATCCA GC. JNK expression plasmids, Flag-JNK1/pSG5, Flag-JNK2/pSG5 and Flag-JNK3/pSG5, were used as templates. (B-D) Expression profiles of *Jnk* isoforms in NIH3T3 cells which were synchronized by 2-hr Dex treatment. (E, F) Expression profiles of *Dbp* and *Clock* were shown as positive control for the synchronization. The primers used were, *Tbp*-Fw ATGGT GTGCA CAGGA GCCAA G, *Tbp*-Rv TCATA GCTAC TGAAC TGCTG, *Dbp*-Fw AATGA CCTTT GAACC TGATC CCGCT, *Dbp*-Rv GCTCC AGTAC TTCTC ATCCT TCTGT, *Clock*-Fw CCTAT CCTAC CTTGG CCACA CA, and *Clock*-Rv TCCCG TGGAG CAACC TAGAT. (G) Two shRNA constructs, shJNK1 and shJNK2, target the 4 splice variants of JNK1 and JNK2, respectively. A shRNA construct termed shJNK1/2 targets all the 8 variants of JNK1 and JNK2. (H) Evaluation of JNK knockdown by RNAi. NIH3T3 cells were transfected by Flag-JNK1/pSG5, Flag-JNK2, or Flag-JNK3 in combination with shJNK1/pBS-mU6, shJNK2, or shJNK1/2. We used anti-Flag antibody for detection of JNK2/JNK3 and anti-JNK1 antibody for JNK1.

Figure S5: Behavioral activity level in LD and behavioral rhythms in LL conditions.

(A) Spontaneous locomotor activity levels in the last 10 days in LD are analyzed. Open and solid bars indicate activity levels of wild-type (WT) and *Jnk3*-deficient

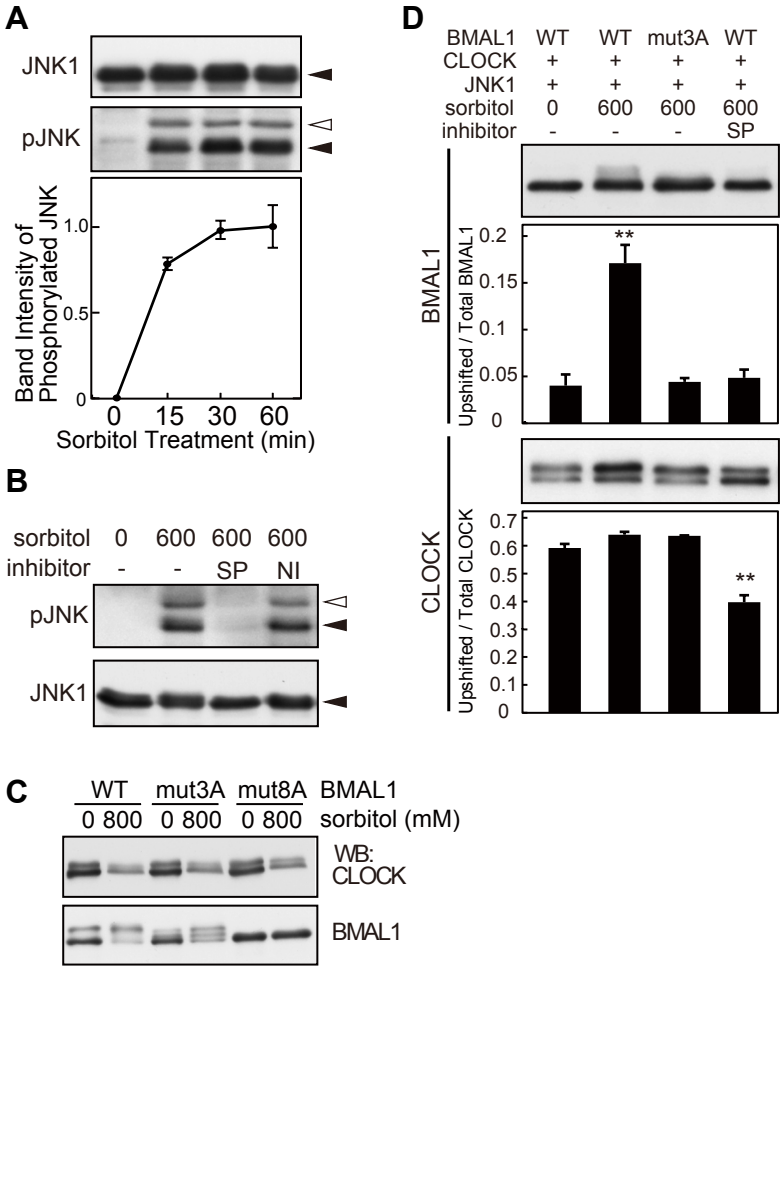
(JNK3KO) mice, respectively. Mean activity levels within the day (Total), those in 12-hr light-phase (Day) and 12-hr dark-phase (Night) are expressed as means with SD of all the mice examined (WT, n = 16; JNK3KO, n = 22). Single and double asterisks indicate $p < 0.05$ and $p < 0.01$, respectively (Student's *t*-test, vs. wild-type). Double-plotted spontaneous locomotor activities of representative wild-type (B) and *Jnk3*-deficient mice (C) under LD 12:12 and constant conditions of 4 different light intensities, 0 (DD), 10, 100 and 300 lux. Shaded (gray) area in the right half of the actogram indicates the dark period, and yellow areas show light periods with different intensities. Horizontal solid and open bars above the actogram indicate the dark and light periods in LD, respectively.

Figure S6: Expression profiles of *Per1* and *Per2* transcripts in the SCN.
The *in situ* hybridization was performed using antisense 45-mer oligonucleotide probes for *mPer1* and *mPer2*, according to the protocol reported previously (6). Shown are the expression profiles of mRNA levels of *Per1* (A) *and Per2* (B) under LD 12:12 condition or *Per1* (C) *and Per2* (D) on the first day of DD in the SCN of wild-type (open circles) or *Jnk3*-deficient mice (solid circles). Significant 24-hr rhythms were detected in both m*Per1* (A, C) and m*Per2* (B, D) expressions (One way ANOVA, $p < 0.01$) in both genotypes, and significant genotype difference was detected for both genes (Two way ANOVA, $p < 0.01$). Data are means with SD (n = 5). Single and double asterisks indicate $p < 0.05$ and $p < 0.01$, respectively (post hoc Tukey Kramer test, vs. wild-type). (E, F) Light-induced changes of *Per1* and *Per2* transcript levels in the SCN of wild-type (WT) and *Jnk3*-deficient (KO) mice were investigated after exposure to 30-min light pulse at CT14 and 22. Constant dark condition (light -) that is also shown in panel g and h are shown as control. ‡: $p < 0.01$ (Student *t*-test, vs. light -). Significant genotype difference was not detected in light-induced mRNA levels of either gene at either ZT.

Figure S7: Expression profiles and dysregulation of *Jnk* transcripts and JNK

proteins in the SCN of *Jnk3*-deficient mice.

The *in situ* hybridization was performed using antisense 45-mer oligonucleotide probes for *Jnk1* (TCCCT CTCAT CTAAC TGCTT GTCCG GGATC TTTGG TGGTG GGGCT), *Jnk2* (TGTCA CTCAT GATGT AGTGT CATAC AGGAT CCTGA GGGCT GCTGC), *Jnk3* (TCTCA TCTAT GGGAA AGGGG TCTTA ACTCA ACAGC GTCTT GCCTG). (A) A representative autoradiograph shows *Jnk3* signals in the SCN of wild-type mouse at ZT2. Scale bar indicates 500 μm. (B-D) Shown are the expression profiles of mRNA levels of *Jnk3* (B), *Jnk1* (C) and *Jnk2* (D) under LD 12:12 condition in the SCN of wild-type (open circles) or *Jnk3*-deficient mice (solid circles). Significant daily rhythms were detected for all the three *Jnk* transcripts (One way ANOVA, $p < 0.01$) in wild-type mice, but not for *Jnk1* in *Jnk3*-deficient mice. Data are means with SD (n = 5). Double asterisks indicate $p < 0.01$ (post hoc Tukey Kramer test, vs. wild-type). (E) The nuclear (Nuc) and cytoplasmic (Cyt) fractions of the SCN punch out were prepared at ZT0, ZT6, ZT12 and ZT18 from wild-type mice (WT) and *Jnk3*-deficient mice (KO). Equal amounts of the samples prepared at the four time points were mixed together and subjected to immunoblot analysis with antibodies against JNK1 (top panel), JNK3 (second and third panels) or phospho-JNK (bottom panel). (F) The samples prepared at the four time points were subjected to immunoblot analysis with anti-JNK1 antibody (top and second panels) and anti-JNK3 antibody (third and bottom panels). (G) The cytoplasmic extracts of the total brain prepared at indicated six time points were subjected to immunoblot analysis with anti-JNK1 antibody (top panel), anti-JNK3 antibody (second panel) and anti-β-actin antibody (bottom panel).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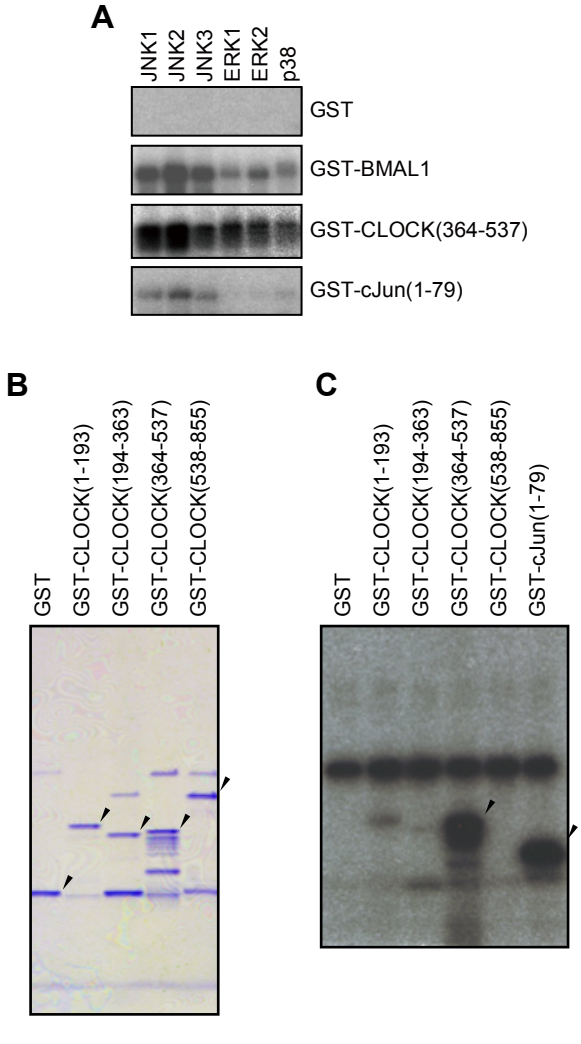
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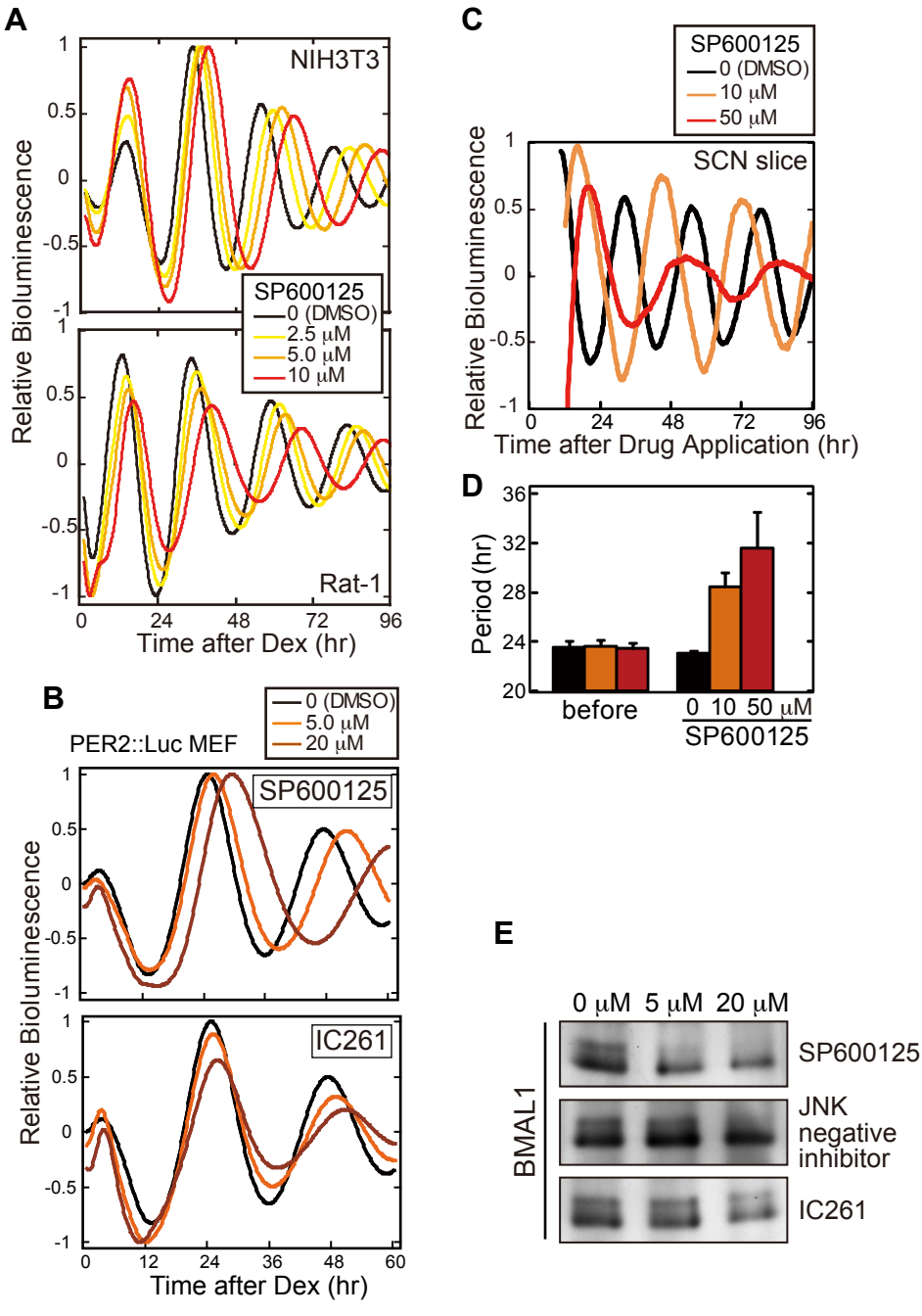
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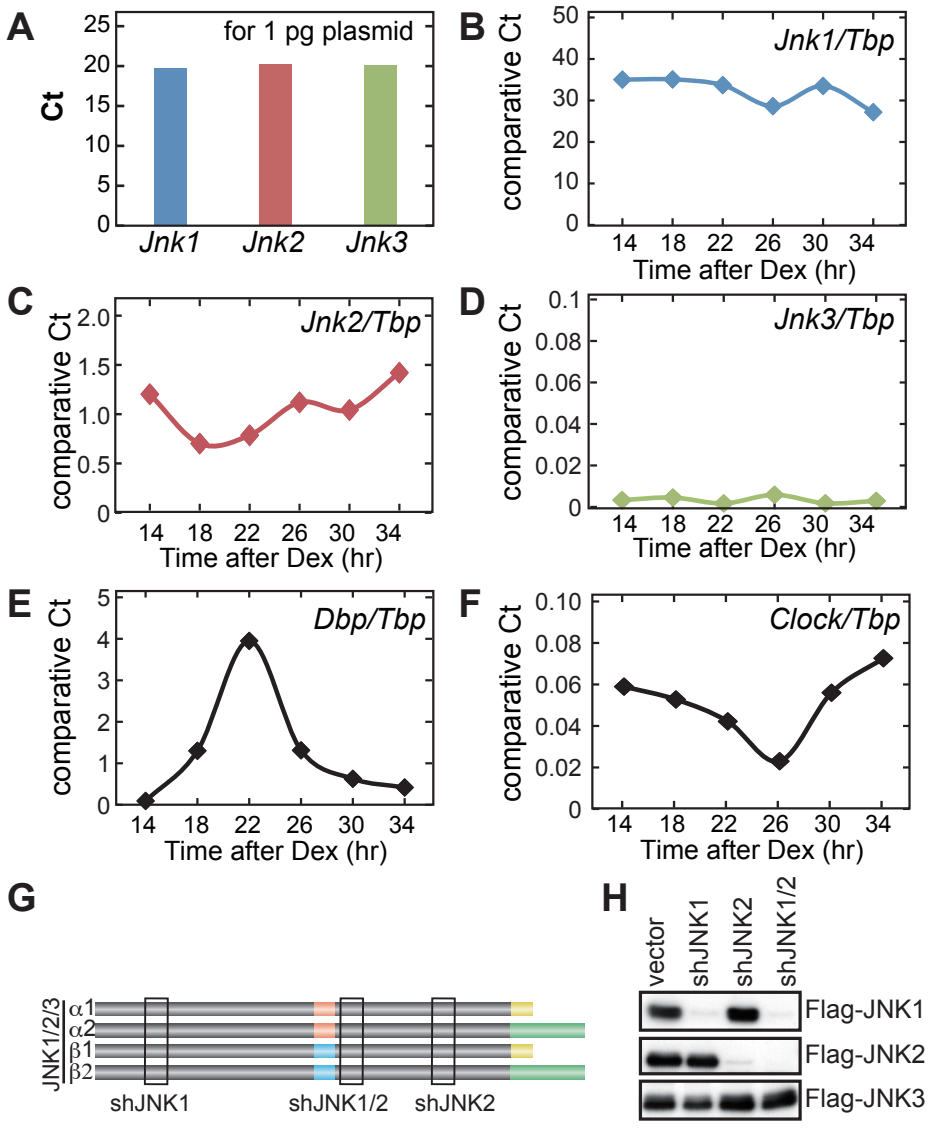
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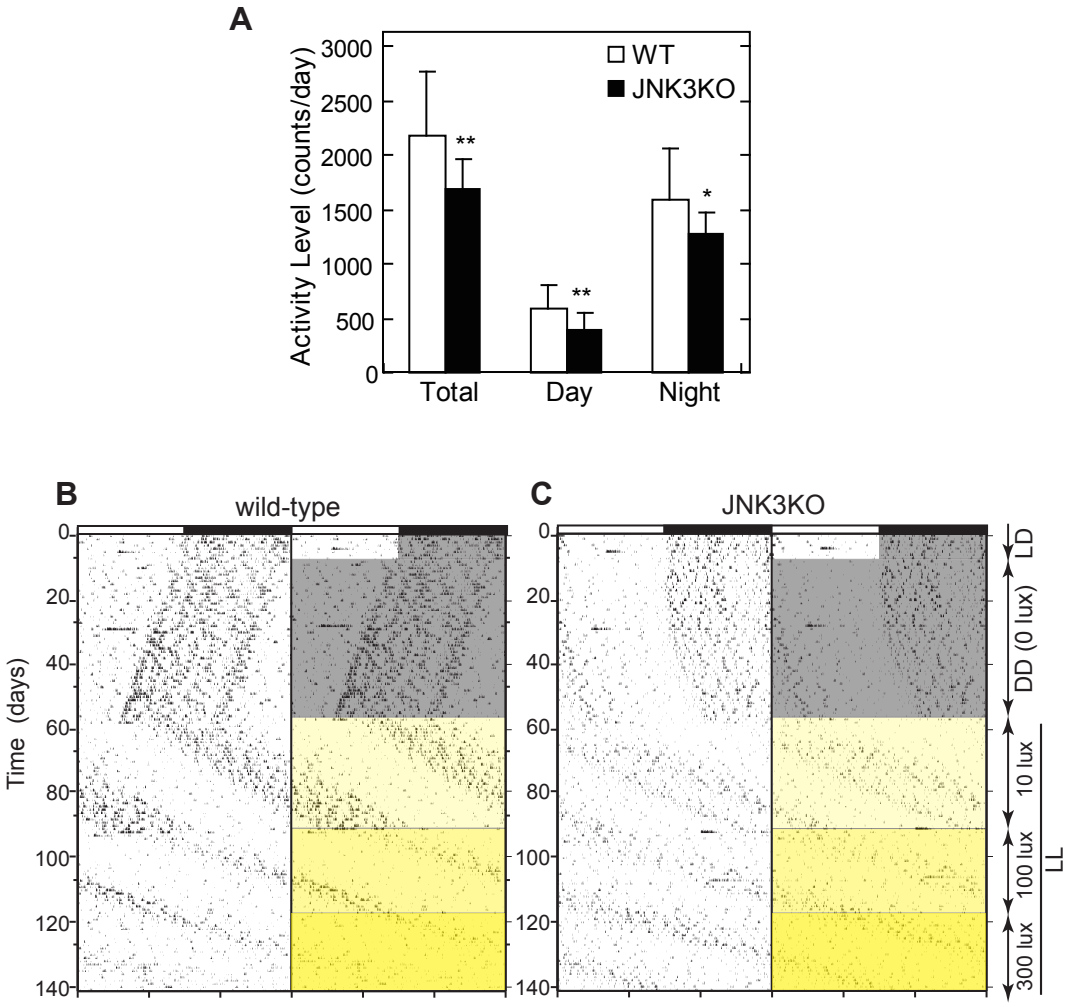
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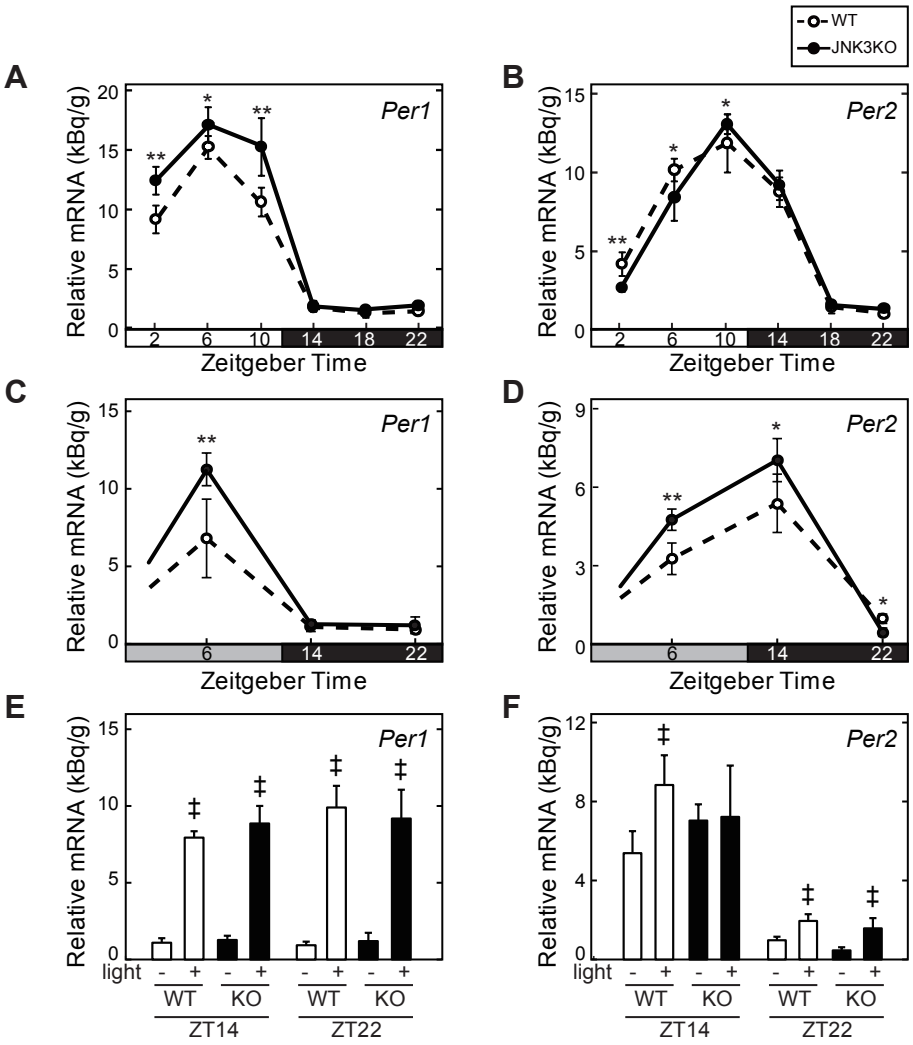
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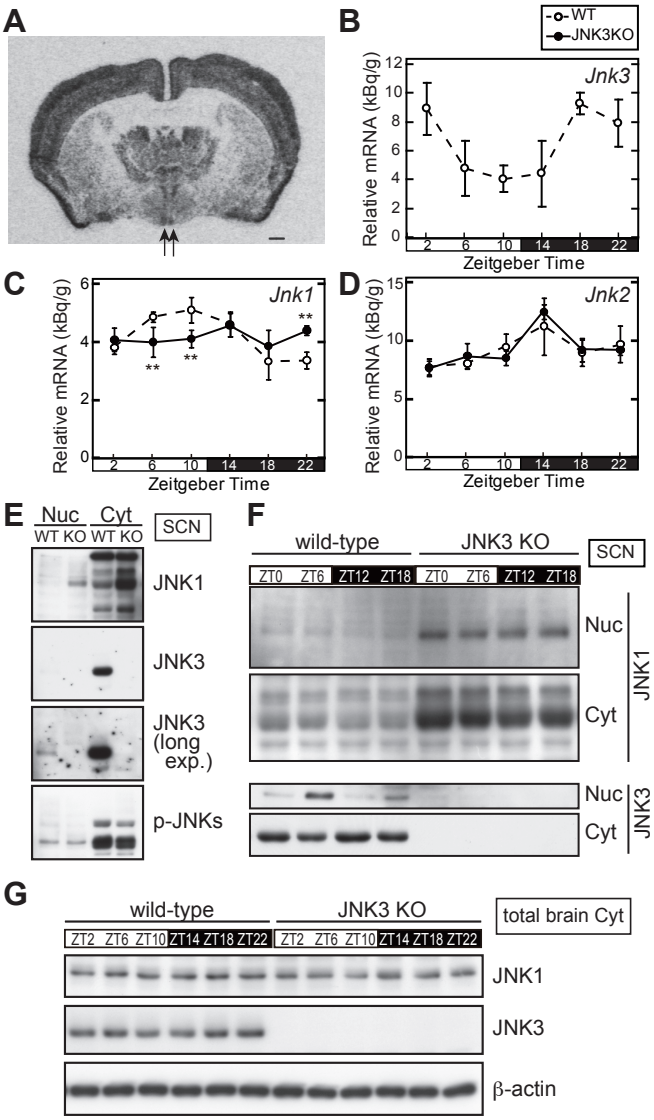
F
WT
JNK3KO
Per1
Per2
Relative mRNA (kBq/g)
Zeitgeber Time
light
WT
KO
ZT14
ZT22


A
B
WT
JNK3KO
Jnk3
Relative mRNA (kBq/g)
Zeitgeber Time
C
Jnk1
D
Jnk2
E
Nuc
Cyt
WT KO WT KO
SCN
JNK1
JNK3
JNK3 (long exp.)
p-JNKs
F
wild-type
JNK3 KO
ZT0 ZT6 ZT12 ZT18 ZT0 ZT6 ZT12 ZT18
Nuc
Cyt
JNK1
JNK3
G
ZT2 ZT6 ZT10 ZT14 ZT18 ZT22 ZT2 ZT6 ZT10 ZT14 ZT18 ZT22
total brain Cyt
β-actin